Activation of the pro-oxidant PKCβII-p66^{Shc} signaling pathway contributes to pericyte dysfunction in skeletal muscles of diabetic patients with critical limb ischemia

Rosa Vono[1] PhD, Claudia Fuoco[2] PhD, Stefano Testa[2] PhD, Stefano Pirrò[2] PhD, Davide Maselli[1] MS, David Ferland Mc Collough[8] PhD, Elena Sangalli[1] MS, Gianfranco Pintus[3,4] PhD, Roberta Giordo[3] PhD, Giovanna Finzi[5] PhD, Fausto Sessa[5] MD, Rosanna Cardani[6] PhD, Ambra Gotti[1] MD, Sergio Losa[1] MD, Gianni Cesareni[2] PhD, Roberto Rizzi[1,7] PhD, Claudia Bearzi[1,7] PhD, Stefano Cannata[2] PhD, *Gaia Spinetti[1] PhD, *Cesare Gargioli[2] PhD and *Paolo Madeddu[8] MD.

[1]IRCCS MultiMedica, Milan, IT;
[2]Department of Biology, University of Rome Tor Vergata, Rome, Italy;
[3]Department of Biomedical Sciences, University of Sassari, Sassari, Italy;
[4]Department of Biomedical Sciences, College of Health Sciences, Qatar University, Doha, Qatar
[5]Department of Pathology, University of Insubria/Ospedale di Circolo, Varese, Italy;
[6]Laboratory of Muscle Histopathology and Molecular Biology, IRCCS-Policlinico San Donato, Milan, Italy;
[7]Cell Biology and Neurobiology Institute, National Research Council of Italy (CNR), Rome, Italy;
[8]Bristol Heart Institute, University of Bristol, UK

Running title: Pericyte dysfunction in diabetic CLI

Word count: 4457
Number of figures: 8
Number of tables: 1

***Corresponding authors**
Prof. Paolo Madeddu, MD
Bristol Heart Institute, School of Clinical Sciences
University of Bristol, Upper Maudlin Road, Bristol BS2 8HW, United Kingdom
tel/fax: 0044 (0)117 342 3904
e-mail: Paolo.Madeddu@bristol.ac.uk

Dr Cesare Gargioli, PhD
Department of Biology
Rome University Tor Vergata
Via della Ricerca Scientifica snc, 00133 Rome, Italy
Tel: +39 0672594815
e-mail: cesare.gargioli@uniroma2.it

Dr Gaia Spinetti, PhD
Laboratory of Cardiovascular Research
IRCCS MultiMedica
Via Fantoli 16/15, 20138 Milan, Italy
Tel: +39-02-55406620
e-mail: gaia.spinetti@multimedica.it

Abstract

Critical limb ischemia (CLI), foot ulcers, former amputation and impaired regeneration are independent risk factors for limb amputation in diabetic subjects. The present work investigates whether and by which mechanism diabetes negatively impacts on functional properties of muscular pericytes (MPs), which are resident stem cells committed to reparative angiomyogenesis.

We obtained muscle biopsies from diabetic patients undergoing major limb amputation and control subjects. Diabetic muscles collected at the rim of normal tissue surrounding the plane of dissection showed myofibres degeneration, fat deposition, and reduction of MPs vascular coverage. Diabetic MPs (D-MPs) display ultrastructural alterations, a differentiation bias towards adipogenesis at the detriment of myogenesis and an inhibitory activity on angiogenesis. Furthermore, they have an imbalanced redox state, with down-regulation of the anti-oxidant enzymes SOD-1 and catalase and activation of the pro-oxidant PKCβII-dependent $p66^{Shc}$ signaling pathway. A reactive oxygen species scavenger or, even more effectively, clinically-approved PKCβII inhibitors restore D-MPs angiomyogenic activity.

Inhibition of the PKCβII-dependent $p66^{Shc}$ signaling pathway could represent a novel therapeutic approach for promotion of muscle repair in diabetes.

Critical limb ischemia (CLI) represents the most severe manifestation of peripheral arterial disease (PAD) and the major cause of foot amputation in the United States (1). Most amputations are carried out on people with diabetes mellitus, who have rampant atherosclerosis and poor angiomyogenesis (2-5).

Satellite cells and pericytes, which reside on opposite sides of the myofibres basement membrane, represent the main myogenic stem/progenitor cells in the postnatal skeletal muscle (6). Satellite cells are well acknowledged targets of diabetes-induced damage and contributors of diabetic vascular myopathy (7-10). Recent evidence indicates pericytes play key role in vascular and muscular regeneration (11-13) and have potential to become favorite candidates for cell therapy of PAD and myocardial ischemia (14-17). However, to the best of our knowledge, no investigation has assessed the impact of diabetes on the functional and molecular makeup of muscular pericytes (MPs).

The mitochondrial adaptor protein Shc1, isoform p66 ($p66^{Shc}$), a redox enzyme that triggers mitochondrial apoptosis, is implicated in the pathophysiology of aging and cardiovascular disease (18-20). Studies in experimental models suggest modulation of $p66^{Shc}$ expression and activity may be a novel and effective target for the treatment of cardiovascular complications. For instance, abrogation of $p66^{Shc}$ results in protection from angiotensin II-induced cardiomyocyte damage (21), improvement of neovascularization and muscle fibres survival following induction of limb ischemia, and preservation of proliferation and differentiation of satellite cells exposed to high oxidative stress (22; 23). Upstream modulators of the $p66^{Shc}$ signaling pathways, such as the protein kinase C, isoform beta-II (PKCβII), which activates $p66^{Shc}$ through phosphorylation of its serine 36 residue at the C-terminal moiety, also represents an attractive therapeutic target to halt ischemic complications (20; 24). Following successful use in diabetic animals, the PKCβII inhibitor LY333531, also known as Ruboxistaurin, has recently shown clear therapeutic benefit in clinical trials of

diabetic microvascular complications (25; 26). However, whether PKCβII inhibitors exert positive effects on myogenic stem cells from diabetic patients with vasculopathy remains currently unknown.

The present study investigates molecular therapeutic targets to improve angiomyogenesis in patients with complicated diabetes. We assessed functional and molecular features of MPs harvested at the rim of tissue above the plane of limb amputation, which is a rescuable area similar to the peri-infarct border zone. We show for the first time that diabetic MPs (D-MPs) are dysfunctional in many respects. They have reduced capacity to expand in culture, differentiate into multinucleated myotubes and support endothelial cells (ECs) network formation. Furthermore, an increased oxidative stress is recurrent in D-MPs, due to the down-regulation of superoxide dismutase 1 (SOD-1) and catalase and up-regulation and activation of $p66^{Shc}$. Importantly, blockade of $p66^{Shc}$ phosphorylation by PKCβII inhibition restores D-MPs functionality, providing a novel indication for molecular treatment of the angiomyogenic pathology in patients with diabetes.

METHODS

Human studies

Skeletal muscle biopsies were obtained from control and diabetic patients following informed consent in line with the guidelines of the Helsinki declaration on human rights. MPs were isolated from: 1) different anatomic districts of the lower extremities from control subjects referring to our Institutions for investigations/therapeutic interventions related to leg varicosity or suspected bone-related pathologies that then resulted negative (n=14) or 2) from sartorius muscles from type-2 diabetic patients at the occasion of major amputation for CLI (n=18). CLI was diagnosed according to TASC 2007, i.e. rest pain and/or ulcer or gangrene, transcutaneous oximetry at the dorsum of the foot <30 mmHg and/or ankle pressure <70 mmHg. Patients' characteristics are listed in **Table 1**. We attempted performing all the analyses on the same sample. When this was not possible due to the small size of the harvested tissue, priority was given to histological analyses and then to the proliferation assay. **Online Supplemental Table 1** summarizes samples attribution to different assays.

Muscle immuno-histochemistry

Morphometric analysis was performed on Haematoxylin & Eosin stained sections. For capillary analysis, sections were boiled in sodium citrate buffer pH 6.0, incubated with mouse monoclonal anti-CD31 antibody clone CJ70A (1:200, Dako), then washed and incubated with secondary antibody followed by DAB+ substrate 1:50 (Kit Dako REAL EnVision). Analysis of inflammatory infiltrates employed rabbit monoclonal anti-CD3 (clone 2GV6) and mouse monoclonal anti-CD68 (clone PG-M1) antibodies (both from Ventana). To localize MPs within the muscle structure, cryosections were treated with Alkaline Phosphatase (ALP) staining solution containing nitro-blue tetrazolium chloride (160ug/mL) and 5-bromo-4-

chloro-3'-indolyphosphate p-toluidine salt (300ug/mL; both from Sigma-Aldrich) in ALP buffer (100mM TrisHCl, 150mM NaCl, 1mM $MgCl_2$ pH 9.0). The same solution was used to stain isolated cells previously fixed in 2% formalin. ALP quantification in cryosections was carried out using Cell Profiler open source software distributed under GPLv2 public license.

Myofibres Cross-Sectional Analysis

Cryosections from diabetic and control muscle biopsies were stained with anti-laminin antibody and analyzed using an ImageJ macro. For each sample (n=3), the area of more than two thousand single fibres was measured.

MP isolation

Human MPs were isolated following well-established procedures (12; 27). Briefly, muscle biopsies were finely minced and digested with collagenase II (100U/mL) for 45min at 37°C on shaking. The digestion mixture was centrifuged and re-suspended in growth medium (α-MEM supplemented with 20% FBS). The cell suspension was filtered through a 70μm cell strainer, dispensed in plastic dishes at clonal density (1000 cell/cm^2) and incubated at 37°C and 5% CO_2 in the growth medium. MPs were selected by plastic adherence in culture for at least 10 days when they form colonies positive for ALP, NG2 and CD146 (27).

Transmission electron microscopy (TEM)

For ultrastructural analysis, a pellet of MPs was fixed for 2h at 4°C in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.05mol/L pH7.3 cacodylate buffer, post-fixed in 1% osmium tetroxide, and embedded in Epon-Araldite. Thin sections were counterstained with uranyl acetate and lead citrate and examined with a Philips/FEI Morgagni electron microscope.

Immuno-cytochemistry

After fixation with 4% paraformaldehyde, cells were permeabilized with 0.3% Triton X-100 in PBS 1X + 1% BSA, then blocked with goat serum 10% in PBS 1X and incubated with polyclonal rabbit anti-NG2 (Merk Millipore), mouse monoclonal anti-CD146 (clone OJ79c, Abcam), mouse monoclonal anti-desmin (clone D33, Dako), mouse monoclonal anti-MyHC (clone MF20, DSHB), mouse monoclonal anti- Ser36-phospho-p66^{Shc} (clone 6E10, Abcam), diluted following manufacturer's instructions. Cells were further incubated with goat anti-mouse or anti-rabbit fluorescent secondary antibodies (Alexa Fluor 488 or 555, Life Technologies). Nuclei were counterstained with DAPI (PanreacAppliChem). Microphotographs were acquired using the imaging software AxioVision Imaging System (Zeiss). When required for 3D image acquisition, an Olympus FV 1000 confocal laser scanning microscope with 60X oil immersion lens was used.

MP flow cytometry

MPs were stained for surface antigen expression using the following antibodies: CD44-APC, CD90-APC, and ALP-PerCP Cy5.5 (all from BD Biosciences). For Ser36-phospho-p66^{Shc} quantification, cells were fixed and permeabilized using BD Citofix/Citoperm kit (BD Biosciences). They were then incubated with anti-Ser36-phospho-p66^{Shc} (Abcam), followed by Alexafluor 633 conjugated secondary antibody. Fluorescence was analyzed on a FACSCanto flow cytometer using the FACSDiva software (BD Biosciences) setting a non-labelled population as a negative control.

Myogenic differentiation

MPs were seeded at 10^5 cells/well in 2-well glass chamber slides and expanded in culture for 7 days to allow cell fusion and myotube formation. Cells were fixed with 4% PFA and permeabilized with 0.3% Triton X-100 1% BSA. Myogenic differentiation and myotube formation were assessed by staining cells with anti-myosin heavy chain antibody (MyHC, clone MF20, DSHB), followed by Alexafluor-555 conjugated secondary antibody.

Adipogenic differentiation

MPs were induced to differentiate into adipocytes using an inductive medium for 4 days, i.e. DMEM GlutaMAX high-glucose, 1% penicillin and streptomycin, 1% Sodium Pyruvate supplemented with 20% FBS, and insulin (1µg/mL). Adipogenic differentiation was assessed by Oil Red staining (Sigma-Aldrich) following PFA fixation.

Proliferation assay

MP proliferation was assessed by ELISA BrdU colorimetric assay kit (Roche). Briefly, cells (10^3 cells/well in a 96-well microplate in triplicate) were grown for 24h and then treated with BrdU (10µM final concentration) for additional 24h. The colorimetric reaction was stopped by adding H_2SO_4 (250mmol/L final concentration) and read immediately at 450 nm.

EC network assay

Human Umbilical Vein ECs (HUVECs) and MPs were seeded in an 8-well permanox chamber slide (Nunc, USA) coated with Matrigel (3D; BD Biosciences) alone ($3.75x10^4$ cell/well) or co-cultured at 1:4 ratio (MPs to HUVECs) in EBM medium, supplemented with 0.1% BSA. Cells were incubated for 5h post-seeding to allow network formation. Pictures of the network were taken on an inverted phase-contrast microscope (AxioObserverA, Zeiss, Germany) and network formation was assessed by counting the number of branches per field.

Similar angiogenesis assays were carried out to assess the effect of MP-derived paracrine factors, by adding Conditioned Culture Media (CCMs) to HUVECs seeded onto Matrigel. In a subset of these studies, the influence of MPs-CCM on *in vitro* angiogenesis was assessed in the presence of high oxidative stress, by adding 300μmol/L H_2O_2 to the media. All experiments were performed in duplicate.

ELISA of MPs-CCM

Levels of VEGF-A, IGF-1, MCP-1, SPARC, HGF, FGF and Ang-2 in MPs-CCM were measured by enzyme-linked immunosorbent assay (ELISA) using Duo Set kits (R&D/Bio-Techne). Levels of Ang-1 were analyzed using the human angiopoietin-1 ELISA kit (Sigma-Aldrich). All analyses were performed following the manufacturer's instructions in triplicate (25ul of sample per replicate).

Studies of MPs-derived exosomes

Exosomes were isolated by sequential ultracentrifugation from MPs-CCM collected after 40h growth in a medium added with 20% exosome–free FBS as previously described (28; 29). Exosome-like microparticle abundance was revealed through Nano Tracking Analysis (NTA), and aliquots of the same preparation were added to HUVECs in the Matrigel assay.

HUVEC redox state

HUVEC intracellular redox state following treatment with MPs-CCM was investigated by using a lentiviral vector encoding for the *redox-sensing green fluorescent protein* (roGFP), which reports the GSH/GSSG balance (30). After overnight treatment of HUVECs with MPs-CCM, H_2O_2 (100 and 300μmol/L) was added. Fluorescence measurements were performed in clear 24-well plates (Corning, Lowell, MA) on a fluorescence plate reader GENios plus

(Tecan, Männedorf, CH) using a 535nm emission filter. The degree of oxidation of the roGFP was estimated from the ratios of light intensities obtained during 1min intervals under 400 and 485nm excitation.

ROS analysis

ROS production by MPs was evaluated labeling cells with MitoSox (Invitrogen) according to the manufacturer's instructions. Briefly, semi-confluent MPs were treated for 10min with 5µmol/L MitoSox dye in culture. Fluorescence was analyzed using FACSCanto flow cytometer and the FACSDiva software (BD Biosciences). In inhibitory studies, the scavenger N-acetyl-cysteine (NAC 1mmol/L, Sigma-Aldrich) was added to MPs to infer the impact of ROS on MPs functions

PKCβII inhibition

Functional assays were performed on MPs treated with the PKCβII inhibitors LY333531 (200nmol/L) or CGP53353 (2µmol/L) or vehicle. Pharmacological inhibition was extended for 7 days in the myogenic differentiation assays and 24h in the proliferation and angiogenesis assays.

Gene expression analysis

RNA was extracted using miRNAeasy extraction kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized using TaqMan Reverse Transcription kit and then analyzed by QuantStudio™ 6 Flex Real-Time PCR System, normalizing the data to the 18S ribosomal RNA (18S rRNA). Primers for the CH2 domain of the p66 isoform were designed as follows:

5’GATCCCGAATGAGTCTCTGTCATCGTTGATATCCGCGATGACAGAGACTCATTC TTTTTTCCAAA-3’

and

3’GGCTTACTCAGAGACAGTAGCAACTATAGGCGCTCTGTCTCTGAGTAAGAAAA AAGGTTTTCGA-5’

Western Blot

Cells were harvested after overnight serum starvation in lysis buffer (TRIS-HCl 20mmol/L, NaCl 150mmol/L, EDTA1mmol/L, EGTA1mmol/L, Triton X-100 1%) supplemented with 1X COMPLETE protease inhibitor cocktail (Roche), 1X phosphatase inhibitor cocktail type II and III (Sigma-Aldrich) and 1X Benzonase (Novagen). Twenty-five micrograms of total cell lysates were loaded onto a NuPAGE 10% Bis-Tris precast gel (Life Technologies). Proteins were then transferred onto an Amersham Hybond PVDF membrane (GE Healthcare, 0.45µm). Blocked membranes with 5% skim milk in 0.1% PBS-TWEEN20 were decorated with mouse monoclonal primary antibodies diluted according to the manufacturer’s instructions. Membranes were subsequently incubated with HRP conjugated anti-mouse secondary antibody (1:1000 in 5% skim milk in 0.1% PBS-TWEEN20). Bands were revealed using the C-Digit blot scanner (LI-COR Biosciences).

Statistical analysis

Continuous variables were expressed as mean±standard error (SEM) and compared using parametric tests (t-test and ANOVA) unless not normally distributed. A p-value <0.05 was considered statistically significant. The GraphPad Prism 5 software package was used for these analyses.

RESULTS

Negative impact of diabetes on muscle anatomy and pericyte abundance

A comparative analysis of muscle specimens from control and diabetic patients was carried out to assess myofibres organization and vascular cell structure. As illustrated in **Figure 1A, left panel,** muscle sections from diabetic patients show abundant and enlarged adipocytes (black star) and myofibres featuring degeneration (arrow) and nuclear centralization (arrowhead). Also, the average myofibre cross-section area was shifted to lower values in the diabetic samples compared with controls (**Figure 1A, right panel**). Moreover, we observed an inflammatory infiltrate in diabetic samples as evidenced by the abundance of CD3-positive lymphocytes and particularly CD68-positive macrophages (**Figure 1B**), which is in line with a previous investigation of ischemic muscles (31). Additionally, the count of CD31-positive capillaries revealed a significant decrease in muscle vascularization (**Figure 1C**).

MPs embracing CD31-positive capillary ECs were identified by immunocytochemistry for NG2 (**Figure 1D**) and ALP (**Figure 1D**, **Supplementary Figures S1A and B**). An unbiased profiler analysis carried out using the Cell Profiler software (32), indicates a remarkable reduction in the density of ALP-positive MPs in diabetic muscles (**Figure 1D lower panel**).

Diabetic MPs show ultrastructural alterations but maintain typical antigenic markers

TEM analyses indicate ultrastructural alterations of D-MPs, consisting of blebbing and vacuolation (**Figure 2A**). Immunocytochemistry studies confirm the pericyte identity of freshly isolated cells, based on the expression of ALP, CD146, and NG2 (**Figure 2B and C**). Expanded MPs express the same markers while being negative for the satellite cell marker PAX7 (**Figure 2D**). Moreover, expanded MPs co-express NG2 and ALP (**Figure 2E**). Flow cytometry analyses indicate the statement of mesenchymal markers CD44 and CD90 (**Figure**

2F). MPs from control or diabetic patients did not differ from each other about antigen expression except desmin, which was down-regulated in D-MPs (**Supplementary Figure S2A-C**).

Diabetic MPs display angiomyogenic deficits

High confluence MPs differentiate and fuse forming myosin heavy chain (MyHC)-positive syncytial myotubes (33; 34). As shown in **Figure 3A,** D-MPs have reduced capacity to generate MyHC-positive myotubes as compared to control MPs (C-MPs), which may be ascribed in part to decreased proliferation, resulting in delayed cell confluence (**Figure 3B**). Additionally, we found that D-MPs have an increased propensity to differentiate into adipocytes (1.7±0.8 fold increase compared with C-MPs, n=3, **Supplementary Figure S2D**). In contrast, diabetes did not affect apoptosis, as assessed by caspase activity (6.7±2.2 vs. 7.3±2.4 RFU at 499/521 nm in controls) or cell motility in a scratch assay at both 5h (8.7±3.7 vs. 3.6±1.7 % wound closure in controls) and 24h post-scratch (60.0±13.8 vs. 43.4±17.4% in controls).

We next analyzed the ability of MPs to form networks on Matrigel. Both C-MPs and D-MPs were able to form branched structures. In comparison with networks formed by HUVECs, the MPs structures consisted of longer tubes and wider meshwork (**Figure 3C**). When co-cultured with HUVECs, MPs locate near the intersection points or around the endothelial tubes (**Figure 3D,** white arrow and arrowhead). Importantly, D-MPs have detrimental effects on network formation, resulting in a less reticulated system (**Figure 3E**).

Paracrine deficits of diabetic MPs

To investigate if paracrine mechanisms are responsible for the negative impact of D-MPs on angiogenesis, we next tested the effect of MPs-conditioned media (MPs-CCM) on HUVECs

in the Matrigel assay. As shown in **Figure 4A**, the D-MPs-CCM induces a decrease in network formation, suggesting and alteration of the secretome. To verify this possibility, we interrogated secreted angiogenic myokines. ELISA showed significantly decreased levels of pro-angiogenic factors, i.e. VEGF-A, IGF-1, and Ang-1, in D-MPs-CCM (**Figure 4B**).

Specific micro-RNAs (miRs) have recently been shown to regulate angiogenesis and their deregulation may contribute to vascular complications and ischemia (29; 35-37). Data from PCR analysis did not show any difference in the expression of classical angiomiRs, miR-16, miR-503 and miR-27b, between D-MPs and C-MPs (**Supplementary Figure S3A**). Also, the analysis of CCMs confirmed D-MPs and C-MPs secrete similar amounts of miR-16, while miR-503 and miR-27b were undetectable (**data not shown**). Likewise, no difference was observed in the expression of the miR-27b targets thrombospondin-1 (TSP-1), sprouty-2 (SPRY-2) and semaphorin 6A (SEM6A) (**Supplementary Figure S3A-ii and B**), which are negative modulators of angiogenesis (36).

Secreted exosomes mediate the functional cross-talk between pericytes and ECs (29; 37; 38). Therefore, we next investigated if diabetes impacts on the MPs capacity to release exosomes or modifies exosomes activity on angiogenesis. Results indicate no difference in exosome-size vesicles shed by C-MPs and D-MPs ($7.4\pm2.5x10^{-4}$ vs. $6.9\pm2x10^{-4}$ exosome concentration/cells, respectively). Also, the addition of exosomes from D-MPs to HUVECs in a Matrigel assay does not alter the network formation (10.7±1.9 vs. 10.8±0.4 intersections/field in HUVECs+C-MPs), while exosome-depleted D-MPs-CCM still inhibits the process (**data not shown**).

We next investigated the role of ROS as paracrine mediators of the negative cross-talk between D-MPs and ECs. To this end, HUVECs were transduced with lentiviral vectors carrying the cytosolic or the mitochondrial isoforms of roGFP, which act as fluorescent indicators of the intracellular redox status, (30) and were then incubated with C-MPs CCM or

D-MPs CCM, before being exposed to increasing doses of the pro-oxidant H_2O_2. We found that C-MPs-CCM protects HUVECs from the pro-oxidant action of H_2O_2, whereas D-MPs-CCM does not (**Figure 4C**). In additional experiments, we test if ROS *per se* impairs angiogenesis *in vitro*. To this end, HUVECs were exposed to a fixed dose of H_2O_2 (300µmol/L) before being seeded into the Matrigel assay. As expected, H_2O_2 inhibited the HUVEC network formation capacity in a way comparable to the inhibition caused by D-MPs or their CCM. Interestingly, the addition of C-MPs-CCM contrasted the inhibitory effect of H_2O_2 on network formation, whereas the D-MPs-CCM was ineffective (**Figure 4D**).

ROS blockade reverts functional deficits of diabetic MPs

We next investigated the redox status of C-MPs and D-MPs by staining cells with MitoSox, a dye that selectively reacts with superoxide anion $O_2^{\bullet}$ in mitochondria. Results of flow cytometry analyses indicate that D-MPs have remarkably increased oxidative stress levels as compared with C-MPs, either considering the average MitoSox dye intensity or number of MitoSox-positive cells (**Figure 5A**). Moreover, the ROS scavenger system involved in the maintenance of cellular redox balance was depressed, as indicated by the down-regulation of SOD-1 and catalase (**Figure 5B**). To confirm that the ROS imbalance contributes to D-MPs dysfunctions, we investigated if the addition of NAC, a generic antioxidant, restores inherent and paracrine activities of the diabetic cells. In line, NAC partially amended D-MPs myogenic differentiation capacity (**Figure 6A**), restored D-MPs proliferation (**Figure 6B**), and abrogated the inhibitory effect of D-MPs-CCM on HUVECs network formation *in vitro* (**Figure 6C**). Since the excess of ROS activates the $p66^{Shc}$ signaling pathway by its selective phosphorylation, the next step was to compare the levels and phosphorylation state of this protein in C-MPs and D-MPs.

Implication of p66^{Shc} in the redox imbalance of D-MPs

Using primers specifically designed for the p66 isoform of Shc1 protein, we found that p66^{Shc} mRNA expression is significantly up-regulated in D-MPs as compared with C-MPs (**Figure 7A**). Under unbalanced redox conditions, p66^{Shc} is phosphorylated at the Ser36 of its unique CH2 domain and translocates to the mitochondrial transmembrane space, where it fuels additional ROS production, causing the formation of a permeability-transition pore and apoptosis. We analyzed the phosphorylation state of p66^{Shc} in MPs by different methods. Using a specific western blot Ser36-p-p66^{Shc} antibody, we observed an increase in the band of activated p66^{Shc} with no difference in the total protein content (**Figure 7B**). Flow cytometry (**Figure 7C**) and immunocytochemistry (**Figure 7D**) confirmed the increased statement of phospho-p66^{Shc} in D-MPs. Nonetheless, treatment of D-MPs with NAC was ineffective in restoring p66^{Shc} phosphorylation to control levels (**Supplementary Figure S4A and B**). This result has different keys of interpretation. First, the thiol groups of the NAC molecule may undergo auto-oxidation processes especially in culture, which reduces their activity. Thus, a possible explanation is that NAC did not reach biologically relevant concentrations in mitochondria, where active p66^{Shc} accumulates. Alternatively, p66^{Shc} activation by PKCβII may be partially independent of ROS or resistant to temporary ROS reduction. The latter possibility is compatible with a reported epigenetic activation of the p66^{Shc} promoter in the context of diabetes (39). Also, ROS scavenging by NAC may be insufficient to contrast the activity of protein kinases responsible for p66^{Shc} phosphorylation, namely protein kinase C βII (PKCβII) (40). Accordingly, we found that NAC supplementation does not affect PKCβII localization or expression (**Supplementary Figure S4C and D**).

Suppression of p66^{Shc} activity by PKCβII inhibition reverts functional deficits of D-MPs

To assess the direct involvement of $p66^{Shc}$ in D-MPs dysfunction, $p66^{Shc}$ activation was blocked by interfering with its phosphorylation. To this purpose, we inhibited the PKCβII kinase, which is responsible for $p66^{Shc}$ activation (39). Two compounds were used to inhibit PKCβII in D-MPs: LY333531, also known as Ruboxistaurin already tested for therapeutic efficacy in clinical trials of diabetic retinopathy, and CGP53353.(41) Flow cytometry confirmed that both LY333531 and CGP53353 reduce the levels of Ser36-phospho-$p66^{Shc}$ (**Figure 8A**). We next re-assessed the features of D-MPs dysfunction following treatment with PKCβII inhibitors. Results indicate an overall functional improvement upon PKCβII inhibition, including myogenic differentiation (**Figure 8B**), proliferation (**Figure 8C**) and interference with network formation (**Figure 8D**) Those data indicate that blocking the PKCβII- $p66^{Shc}$ pathway may have important therapeutic implications for total restoration of D-MPs functions.

DISCUSSION

The present study is the first to investigate the anatomical, functional and molecular diversity of MPs from skeletal muscles of diabetic patients with CLI. Importantly, MPs were isolated from the tissue immediately at the rim of normal tissue surrounding the plane of dissection, a critical zone where it is mandatory to concentrate efforts for limb salvage. Results show for the first time specific alterations consisting of ultrastructural modifications, proliferative impairment leading to blunted myogenic potential, and acquisition of an anti-angiogenic activity. Altogether, these deficits could contribute to the extension and severity of peripheral complications. Importantly, we discovered that diabetes-associated MPs incompetence is attributable to increased ROS levels, weakened anti-oxidative protection, and activated PKCβII-$p66^{Shc}$ signaling pathway. Also, excessive ROS production and release transmit negative signals to adjacent vascular cells. Muscle regeneration *via* trans-differentiation of MPs and satellite cells into myoblasts is crucial for the recovery of damaged muscles, whereas overgrowth of adipogenic cells may be deleterious. Some studies suggest that a subset of MPs contribute to fat accumulation (42). Our investigation shows structural data that are compatible with a differentiation bias of MPs favoring adipogenesis at detriment of myogenesis. Concurrent mechanisms may participate in this adverse remodeling, including poor metabolic control, ischemia, and lack of exercise (43).

Unraveling the molecular mechanisms underpinning MPs dysfunction could help develop new strategies to maintain tissue integrity and improve clinical outcomes especially after major limb amputation, a condition associated with high mortality. Current mechanistic understanding of diabetic vasculopathy is mainly inferred from animal models (44). However, the clinical transferability of data from rodents mimicking human diabetes/CLI is limited and often equivocal. Therefore, the successful isolation of MPs from muscles of diabetic patients undergoing major limb amputation for CLI discloses excellent opportunities

for disease modeling and therapeutics. Since there is a potential overlapping between MPs and satellite cells, it was essential to ascertain that isolated cells express high levels of the canonical pericyte marker NG2 and ALP along with mesenchymal markers (CD90 and CD44), the staminal marker CD146, while being negative for PAX7. In fact, ALP positivity and PAX7 negativity are determinants to distinguish pericytes from $ALP^-/PAX7^-$ mature myocytes or $ALP^-/PAX7^+$ satellite cells. When grown to over-confluence, MPs spontaneously fuse together to form syncytial myotubes expressing MyHC. Interestingly, the ability to form syncytial myotubes was drastically reduced in D-MPs, and this could be one of the reasons (together with satellite cell dysfunction) of the aberrant tissue repair of diabetic skeletal muscles.

Skeletal muscle physiology is maintained by mutual trophic influences between MPs, ECs and myocytes (45). However, this cross-talk is perversely modified by diabetes. The present investigation integrates results of our recent study showing that p75NTR expression in ECs exposed to high glucose activates the transcription of miR-503, which negatively affects pericyte function (37; 46). Here, we newly show that D-MPs exert a drastic reduction of ECs ability to form *in vitro* networks through an alteration in the secretion of angiogenic growth factors. Instead, screening of several classical angiomiRs or exosomes did not provide any supplementary clue.

An additionally accountable mechanism emerged from studies of the MPs redox state and was confirmed by the recognition of a reduced scavenging capacity of D-MPs, consisting of down-regulation of SOD-1 and catalase gene expression. Accordingly, restoration of the physiologic redox state by NAC supplementation led to the correction or attenuation of D-MPs dysfunctions. A more in-depth analysis of the original culprit directed us to recognize the intracellular stress sensor as $p66^{Shc}$ protein. Several lines of evidence support this possibility: (i) $p66^{Shc}$ is up-regulated at mRNA level and (ii) and hyper-phosphorylated at the

Ser36 residue, which corresponds to an activated pro-oxidant state of $p66^{Shc}$. However, NAC administration did not revert $p66^{Shc}$ activation so other targets are needed to suppress $p66^{Shc}$ activation. Previous studies in animal models employed the abrogation or silencing of $p66^{Shc}$ to infer its pathophysiological importance. However, this approach has translational limitations as $p66^{Shc}$ is thought to have bivalent actions (47). Thus, its total suppression may not be therapeutically desirable. Therefore, we decided to interfere with the excessive phosphorylation of $p66^{Shc}$ by inhibiting PKCβII (39; 41). Importantly, the use of PKCβII specific inhibitors, namely LY333531 or CGP53353, reverted $p66^{Shc}$ phosphorylation and restored D-MP functions. Even though NAC has been efficiently used to inhibit PKCβII expression in other experimental settings, dosages and cell type specificity may explain the observed differences (48; 49). Moreover, NAC is susceptible to auto-oxidation in culture conditions reducing its effectiveness. Importantly, PKCβII inhibitors lead, at least *in vitro*, to a more effective rescue of D-MPs myogenic differentiation capacity compared to NAC treatment, thus warranting additional investigation in patients with limb ischemia. This may be clinically relevant since generic antioxidant therapy has given disappointing results in trials assessing vitamins supplementation efficacy for cardiovascular diseases (50).

In conclusion, we demonstrate that (i) MPs can be efficiently isolated from skeletal muscles of non-diabetic and diabetic patients; (ii) D-MPs are dysfunctional in terms of reduced myogenic ability, decreased proliferation and anti-angiogenic properties; (iii) those alterations are strictly related to an increased oxidative status driven by $p66^{Shc}$ over-expression and activation; (iv) anti-oxidant treatment as well as $p66^{Shc}$ phosphorylation blockade by inhibition of PKCβII can rescue D-MP functional competence.

These results are important since one of the used PKCβII inhibitors, LY333531, is currently at the final step of clinical trial investigation (phase 3) in patients with diabetic retinopathy. This opens invaluable opportunities for the treatment of life-threatening

complications of diabetes. We hypothesize that the use PKCβII inhibitors may halt the progression of CLI and allow surgeons to decide for less extensive amputations, when these become necessary, ultimately improving the life quality and duration of patients with complicated diabetes.

Acknowledgements

The authors wish to acknowledge Dr. Laura Cantone from EPIGET (Epidemiologia, Epigenetica e Tossicologia) Lab Dipartimento di Scienze Cliniche e di Comunità Università degli Studi di Milano, Milan, Italy for NTA analysis of exosomes, and the centre of advanced microscopy "P. Albertano" at Department of Biology, University of Rome Tor Vergata for confocal images.

Source of Funding: This work has been supported by grants from: the British Heart Foundation program grant number RJ5905 and the Italian Ministry of Health code: RF-2011-02346867 to PM; the Cariplo Foundation, code: 2013-0887 to GS; the European Research Council [N322749 DEPTH] to G.C., and Uncovering Excellence Grant 2014 [MDESMPLAT] to C.G.

Author contribution: V.R. participate to the study design, researched and interpreted data and wrote the manuscript; C.F. and E.S. isolated and characterized human pericytes; S.T. and D.M. performed immunofluorescence and histochemistry analysis; D.F.MC performed measurement of secreted factors by ELISA assay; G.F. and F.S. conducted and analyzed electron microscopy studies; G.P. and R.G. performed redox analyses of endothelial cells; R.C., A.G., and S.L. are responsible for patient enrollment and muscle sample collection; S.P. set and performed Cell Profiler analysis for ALP staining area quantification and statistical analysis; R.R. and C.B. helped with data interpretation; G.C. and S.M.C. helped with study design, data analysis interpretation, and paper writing; C.G. G.S. and P.M. are the guarantors of the article, designed the study, helped with data analysis and interpretation and wrote the manuscript.

Prior publications:

Accepted as a poster at America Heart Association–ATVB Scientific Session 2015-Orlando, FL, USA. Abstract Number: 2015-SS-A-19258-AHA; Accepted as a poster at ESC CONGRESS 2015, London, UK. Abstract Number: 90003; Accepted as a poster at ESM-EVBO Joint Meeting 2015, Pisa, Italy. Abstract Number: 46640.

Conflict of interests: No conflicts of interest relevant to this article were reported

REFERENCES

1. Selvin E, Erlinger TP: Prevalence of and risk factors for peripheral arterial disease in the United States: results from the National Health and Nutrition Examination Survey, 1999-2000. *Circulation* 110:738-743, 2004
2. Nehler MR, Duval S, Diao L, Annex BH, Hiatt WR, Rogers K, Zakharyan A, Hirsch AT: Epidemiology of peripheral arterial disease and critical limb ischemia in an insured national population. *J Vasc Surg* 60:686-695 e682, 2014
3. Faglia E, Clerici G, Clerissi J, Gabrielli L, Losa S, Mantero M, Caminiti M, Curci V, Quarantiello A, Lupattelli T, Morabito A: Long-term prognosis of diabetic patients with critical limb ischemia: a population-based cohort study. *Diabetes Care* 32:822-827, 2009
4. Fadini GP, Boscaro E, de Kreutzenberg S, Agostini C, Seeger F, Dimmeler S, Zeiher A, Tiengo A, Avogaro A: Time Course and Mechanisms of Circulating Progenitor Cell Reduction in the Natural History of Type 2 Diabetes. *Diabetes Care* 33:1097-1102, 2010
5. Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, Hunt TK, Gottrup F, Gurtner GC, Longaker MT: *Human skin wounds: a major and snowballing threat to public health and the economy*, Wound Repair Regen. 2009 Nov-Dec;17(6):763-71. doi: 10.1111/j.1524-475X.2009.00543.x., 2009
6. Relaix F, Zammit PS: Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. *Development* 139:2845-2856, 2012
7. D'Souza DM, Al-Sajee D, Hawke TJ: Diabetic Myopathy: Impact of Diabetes Mellitus on Skeletal Muscle Progenitor Cells. *Frontiers in Physiology* 4, 2013
8. Broholm C, Brandt C, Schultz NS, Nielsen AR, Pedersen BK, Scheele C: Deficient leukemia inhibitory factor signaling in muscle precursor cells from patients with type 2 diabetes. *Am J Physiol Endocrinol Metab* 303:E283-292, 2012
9. Gaster M, Rustan AC, Aas V, Beck-Nielsen H: Reduced lipid oxidation in skeletal muscle from type 2 diabetic subjects may be of genetic origin: evidence from cultured myotubes. *Diabetes* 53:542-548, 2004
10. Scarda A, Franzin C, Milan G, Sanna M, Dal Pra C, Pagano C, Boldrin L, Piccoli M, Trevellin E, Granzotto M, Gamba P, Federspil G, De Coppi P, Vettor R: Increased

adipogenic conversion of muscle satellite cells in obese Zucker rats. *Int J Obes (Lond)* 34:1319-1327, 2010
11. Birbrair A, Zhang T, Wang Z-M, Messi ML, Enikolopov GN, Mintz A, Delbono O: Skeletal muscle pericyte subtypes differ in their differentiation potential. *Stem Cell Research* 10:67-84, 2013
12. Fuoco C, Sangalli E, Vono R, Testa S, Sacchetti B, Latronico MV, Bernardini S, Madeddu P, Cesareni G, Seliktar D, Rizzi R, Bearzi C, Cannata SM, Spinetti G, Gargioli C: 3D hydrogel environment rejuvenates aged pericytes for skeletal muscle tissue engineering. *Front Physiol* 5:203, 2014
13. Zheng B, Chen CW, Li G, Thompson SD, Poddar M, Peault B, Huard J: Isolation of myogenic stem cells from cultures of cryopreserved human skeletal muscle. *Cell Transplant* 21:1087-1093
14. Campagnolo P, Cesselli D, Zen AAH, Beltrami AP, Kränkel N, Katare R, Angelini G, Emanueli C, Madeddu P: Human Adult Vena Saphena Contains Perivascular Progenitor Cells Endowed With Clonogenic and Proangiogenic Potential. *Circulation* 121:1735-1745, 2010
15. Gubernator M, Slater SC, Spencer HL, Spiteri I, Sottoriva A, Riu F, Rowlinson J, Avolio E, Katare R, Mangialardi G, Oikawa A, Reni C, Campagnolo P, Spinetti G, Touloumis A, Tavare S, Prandi F, Pesce M, Hofner M, Klemens V, Emanueli C, Angelini G, Madeddu P: Epigenetic profile of human adventitial progenitor cells correlates with therapeutic outcomes in a mouse model of limb ischemia. *Arterioscler Thromb Vasc Biol* 35:675-688, 2015
16. Katare R, Riu F, Mitchell K, Gubernator M, Campagnolo P, Cui Y, Fortunato O, Avolio E, Cesselli D, Beltrami AP, Angelini G, Emanueli C, Madeddu P: Transplantation of human pericyte progenitor cells improves the repair of infarcted heart through activation of an angiogenic program involving micro-RNA-132. *Circ Res* 109:894-906, 2011
17. Avolio E, Caputo M, Madeddu P: Stem cell therapy and tissue engineering for correction of congenital heart disease. *Front Cell Dev Biol* 3:39, 2015
18. Paneni F, Costantino S, Volpe M, Luscher TF, Cosentino F: Epigenetic signatures and vascular risk in type 2 diabetes: a clinical perspective. *Atherosclerosis* 230:191-197, 2013
19. Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, Pelliccia G, Luzi L, Minucci S, Marcaccio M, Pinton P, Rizzuto R, Bernardi P, Paolucci F, Pelicci PG: Electron transfer between cytochrome c and p66Shc generates reactive oxygen species that trigger mitochondrial apoptosis. *Cell* 122:221-233, 2005
20. Pinton P, Rimessi A, Marchi S, Orsini F, Migliaccio E, Giorgio M, Contursi C, Minucci S, Mantovani F, Wieckowski MR, Del Sal G, Pelicci PG, Rizzuto R: Protein kinase C beta and prolyl isomerase 1 regulate mitochondrial effects of the life-span determinant p66Shc. *Science* 315:659-663, 2007
21. Graiani G, Lagrasta C, Migliaccio E, Spillmann F, Meloni M, Madeddu P, Quaini F, Padura IM, Lanfrancone L, Pelicci P, Emanueli C: Genetic deletion of the p66Shc adaptor protein protects from angiotensin II-induced myocardial damage. *Hypertension* 46:433-440, 2005
22. Zaccagnini G, Martelli F, Magenta A, Cencioni C, Fasanaro P, Nicoletti C, Biglioli P, Pelicci PG, Capogrossi MC: p66(ShcA) and oxidative stress modulate myogenic differentiation and skeletal muscle regeneration after hind limb ischemia. *J Biol Chem* 282:31453-31459, 2007

23. Natalicchio A, Tortosa F, Labarbuta R, Biondi G, Marrano N, Carchia E, Leonardini A, Cignarelli A, Bugliani M, Marchetti P, Fadini GP, Giorgio M, Avogaro A, Perrini S, Laviola L, Giorgino F: The p66(Shc) redox adaptor protein is induced by saturated fatty acids and mediates lipotoxicity-induced apoptosis in pancreatic beta cells. *Diabetologia* 58:1260-1271, 2015
24. Geraldes P, King GL: Activation of protein kinase C isoforms and its impact on diabetic complications. *Circ Res* 106:1319-1331, 2010
25. Danis RP, Sheetz MJ: Ruboxistaurin: PKC-beta inhibition for complications of diabetes. *Expert Opin Pharmacother* 10:2913-2925, 2009
26. Budhiraja S, Singh J: Protein kinase C beta inhibitors: a new therapeutic target for diabetic nephropathy and vascular complications. *Fundam Clin Pharmacol* 22:231-240, 2008
27. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, Bianco P: Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 131:324-336, 2007
28. Thery C, Amigorena S, Raposo G, Clayton A: Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* Chapter 3:Unit 3 22, 2006
29. Spinetti G, Fortunato O, Caporali A, Shantikumar S, Marchetti M, Meloni M, Descamps B, Floris I, Sangalli E, Vono R, Faglia E, Specchia C, Pintus G, Madeddu P, Emanueli C: MicroRNA-15a and MicroRNA-16 Impair Human Circulating Proangiogenic Cell Functions and Are Increased in the Proangiogenic Cells and Serum of Patients With Critical Limb Ischemia. *Circulation Research* 112:335-346, 2013
30. Posadino AM, Cossu A, Giordo R, Zinellu A, Sotgia S, Vardeu A, Hoa PT, Nguyen le HV, Carru C, Pintus G: Resveratrol alters human endothelial cells redox state and causes mitochondrial-dependent cell death. *Food Chem Toxicol* 78:10-16, 2015
31. Fink LN, Costford SR, Lee YS, Jensen TE, Bilan PJ, Oberbach A, Bluher M, Olefsky JM, Sams A, Klip A: Pro-inflammatory macrophages increase in skeletal muscle of high fat-fed mice and correlate with metabolic risk markers in humans. *Obesity (Silver Spring)* 22:747-757, 2014
32. Dellavalle A, Sampaolesi M, Tonlorenzi R, Tagliafico E, Sacchetti B, Perani L, Innocenzi A, Galvez BG, Messina G, Morosetti R, Li S, Belicchi M, Peretti G, Chamberlain JS, Wright WE, Torrente Y, Ferrari S, Bianco P, Cossu G: Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. *Nat Cell Biol* 9:255-267, 2007
33. Peault B, Rudnicki M, Torrente Y, Cossu G, Tremblay JP, Partridge T, Gussoni E, Kunkel LM, Huard J: Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. *Mol Ther* 15:867-877, 2007
34. Nguyen N-U-N, Wang H-V: Dual Roles of Palladin Protein in *In Vitro* Myogenesis: Inhibition of Early Induction but Promotion of Myotube Maturation. *PLoS ONE* 10:e0124762, 2015
35. Meloni M, Marchetti M, Garner K, Littlejohns B, Sala-Newby G, Xenophontos N, Floris I, Suleiman MS, Madeddu P, Caporali A, Emanueli C: Local inhibition of microRNA-24 improves reparative angiogenesis and left ventricle remodeling and function in mice with myocardial infarction. *Mol Ther. 2013 Jul;21(7):1390-402. doi: 10.1038/mt.2013.89. Epub 2013 Jun 18.*, 2013

36. Wang JM, Tao J, Chen DD, Cai JJ, Irani K, Wang Q, Yuan H, Chen AF: MicroRNA miR-27b rescues bone marrow-derived angiogenic cell function and accelerates wound healing in type 2 diabetes mellitus. *Arterioscler Thromb Vasc Biol. 2014 Jan;34(1):99-109. doi: 10.1161/ATVBAHA.113.302104. Epub 2013 Oct 31.*, 2014
37. Caporali A, Meloni M, Vollenkle C, Bonci D, Sala-Newby GB, Addis R, Spinetti G, Losa S, Masson R, Baker AH, Agami R, le Sage C, Condorelli G, Madeddu P, Martelli F, Emanueli C: Deregulation of microRNA-503 contributes to diabetes mellitus-induced impairment of endothelial function and reparative angiogenesis after limb ischemia. *Circulation* 123:282-291, 2011
38. Mayo JN, Bearden SE: Driving the Hypoxia-Inducible Pathway in Human Pericytes Promotes Vascular Density in an Exosome-Dependent Manner. *Microcirculation* 22:711-723, 2015
39. Paneni F, Mocharla P, Akhmedov A, Costantino S, Osto E, Volpe M, Lüscher TF, Cosentino F: Gene Silencing of the Mitochondrial Adaptor p66Shc Suppresses Vascular Hyperglycemic Memory in Diabetes. *Circulation Research* 111:278-289, 2012
40. Mehta NK, Mehta KD: Protein kinase C-beta: An emerging connection between nutrient excess and obesity. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* 1841:1491-1497, 2014
41. Liu Y, Jin J, Qiao S, Lei S, Liao S, Ge Z-D, Li H, Wong GT-C, Irwin MG, Xia Z: Inhibition of PKCb2 overexpression ameliorates myocardial ischaemia/reperfusion injury in diabetic rats via restoring caveolin-3/Akt signaling. *Clinical Science* 129:331-344, 2015
42. Uezumi A, Fukada S, Yamamoto N, Takeda S, Tsuchida K: Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. *Nat Cell Biol* 12:143-152, 2010
43. Rockl KS, Hirshman MF, Brandauer J, Fujii N, Witters LA, Goodyear LJ: Skeletal muscle adaptation to exercise training: AMP-activated protein kinase mediates muscle fiber type shift. *Diabetes* 56:2062-2069, 2007
44. Heinonen SE, Genove G, Bengtsson E, Hubschle T, Akesson L, Hiss K, Benardeau A, Yla-Herttuala S, Jonsson-Rylander AC, Gomez MF: Animal models of diabetic macrovascular complications: key players in the development of new therapeutic approaches. *J Diabetes Res* 2015:404085, 2015
45. Abou-Khalil R, Mounier Rm, Chazaud Bnd: Regulation of myogenic stem cell behaviour by vessel cells: The "ménage à trois" of satellite cells, periendothelial cells and endothelial cells. *Cell Cycle* 9:892-896, 2010
46. Caporali A, Meloni M, Nailor A, Mitic T, Shantikumar S, Riu F, Sala-Newby GB, Rose L, Besnier M, Katare R, Voellenkle C, Verkade P, Martelli F, Madeddu P, Emanueli C: p75(NTR)-dependent activation of NF-kappaB regulates microRNA-503 transcription and pericyte-endothelial crosstalk in diabetes after limb ischaemia. *Nat Commun* 6:8024, 2015
47. Giorgio M, Berry A, Berniakovich I, Poletaeva I, Trinei M, Stendardo M, Hagopian K, Ramsey JJ, Cortopassi G, Migliaccio E, Notzli S, Amrein I, Lipp HP, Cirulli F, Pelicci PG: The p66Shc knocked out mice are short lived under natural condition. *Aging Cell* 11:162-168, 2012

48. Xia Z, Kuo KH, Nagareddy PR, Wang F, Guo Z, Guo T, Jiang J, McNeill JH: N-acetylcysteine attenuates PKCbeta2 overexpression and myocardial hypertrophy in streptozotocin-induced diabetic rats. *Cardiovasc Res* 73:770-782, 2007
49. Liu Y, Lei S, Gao X, Mao X, Wang T, Wong GT, Vanhoutte PM, Irwin MG, Xia Z: PKCbeta inhibition with ruboxistaurin reduces oxidative stress and attenuates left ventricular hypertrophy and dysfunction in rats with streptozotocin-induced diabetes. *Clin Sci (Lond)* 122:161-173, 2012
50. Cook NR, Albert CM, Gaziano JM, Zaharris E, MacFadyen J, Danielson E, Buring JE, Manson JE: A randomized factorial trial of vitamins C and E and beta carotene in the secondary prevention of cardiovascular events in women: results from the Women's Antioxidant Cardiovascular Study. *Arch Intern Med* 167:1610-1618, 2007

TABLE 1: Characteristics of the study populations

	Controls (n=14)	**Diabetic** (n=18)	**p-value**
AGE (years±SEM)	70±3	75±2	0.1
SEX (male, %)	54	67	0.2
Time renown diabetes (Years±SEM)	0	17.2±3.2	
HbA1c % Hb±SEM(mmol/mol±SEM)	n.a.	7.4±0.5 (63.2±4.3)	
Glycemia (mmol/L±SEM)	5.4±0.4	8.8±1.6	0.02
Creatinine (μmol/L±SEM**)**	91.3±11	132.4±35	0.9

FIGURES

Figure 1. In situ characterization of MPs. A) Representative microphotographs of Hematoxylin & Eosin (H&E) stained sections of human muscles. The arrow indicates degenerated fibres, while the arrowhead points at a centralized nucleus. Fat deposition is indicated by the black star. Quantitative analysis depicted in the bar graph. Fibres composition was compared between C-MPs and D-MPs measuring the cross-sectional area (**left panel**). **B**) The presence of inflammatory cells infiltration was quantified by immunohistochemistry using anti-CD3 and CD68 antibodies to detect respectively lymphocytes and macrophages (arrowheads). Macrophages infiltration was significantly increased in D-MPs. *$p<0.05$ vs. controls, n=4. Scale bar 20 µm. **C**) Capillaries in the sections are indicated by brown CD31 positive staining (black arrows) used for the quantification expressed in the bar graph. Values expressed as mean±SEM of 3 control and 9 diabetic subjects; **$p<0.01$ vs. controls. Scale bar 20µm. **D**) Within the muscle structure, pericytes, which encircle capillaries (CD31 positive cells), were identified by NG2 expression (white arrowheads). Nuclei were counterstained with DAPI. Pericyte count in bar graph. *$p<0.05$ vs. control. Scale bar 20µm. In addition, pericytes were identified by positivity for ALP using the Cell Profiler software. The number of ALP-positive pericytes in muscles from diabetic patients was significantly reduced with respect to controls. Values expressed as mean±SEM on 3 different donors; * $p<0.05$ vs. controls. Scale bar 100 µm.

Figure 2. Characterization of isolated MPs. A) Transmission electron microscopy ultrastructure of MPs. Magnification: 7100X. **B**) ALP immunostaining and **C**) NG2 and CD146 immunofluorescence of MPs after isolation from muscle biopsies. Positive cells were purple stained. Scale bar 20µm. **D**) MPs expression of ALP, NG2, CD-146 and PAX7 to assess the antigenic characteristics of isolated cells. Nuclei were counterstained with DAPI.

Scale bar 50μm. **E**) Double staining of MPs with ALP and anti NG2. Scale bar 10μm. **F**) Flow cytometry analysis of MPs. Data showed as peaks of positivity to the staining (red line) for the markers CD90, CD44 and ALP vs. negative control (dark line).

Figure 3. MP differentiation, proliferation and network formation. A) *Muscle differentiation*: MPs were stained with anti-myosin heavy chain (MyHC) antibody to reveal syncytial myofibre-like structures (yellow arrowheads) generated by fusion. Nuclei were counterstained with DAPI. Bar graph summarizes the number of MyHC positive fibres counted on the total number of nuclei per field. Counts were made on 5 pictures from at least 3 biological replicates. Picture magnification 20X, scale bar 50μm. Values expressed as mean±SEM. *$p<0.05$ vs. C-MPs. **B**) *Cell proliferation* was measured as optical density after 48h of culture of which the last 24h in the presence of 5-bromo-2'-deoxyuridine (BrdU) as described in "Methods". Values are expressed as mean±SEM of 4 experiments in triplicate. *$p<0.05$ vs. C-MPs. **C**) *Matrigel assay*: compared to HUVECs (left panel), MPs (right panel) form networks less reticulated with thicker nodes and longer tubes. Scale bar 100 μm. **D**) HUVECs-MPs interaction on Matrigel. Fluorescent microphotograph of networks formed on Matrigel by co-culturing HUVECs (stained red with PKH26 dye) and MPs (stained green with PKH67 dye) at 5x magnification. MPs locate preferentially at nodes of the network (white arrow). They are also located along the tube assisting HUVECs in tubulization (white arrowhead). Scale bar 100μm. **E**) Direct interaction with endothelial cells (HUVECs): microphotographs of networks formed by MPs in co-culture with HUVECs at a 1:4 MPs/HUVECs ratio. Bar graphs represent the efficiency of network formation in terms of number of branches. Values expressed as mean±SEM of at least 5 experiments in duplicate; *$p<0.05$ vs. HUVECs+C-MPs. Scale bar 100 μm.

Figure 4. Paracrine effect of MPs on HUVECs. **A**) Matrigel assay performed with HUVECs at $5x10^4$ cell density in the presence of MPs-CCM. Bar graphs represent the efficiency of network formation (number of branches). Values expressed as mean±SEM of 5 experiments in duplicates. *p<0.05 vs. HUVECs+C-MPs-CCM. Scale bar 100μm. **B**) Myokines regulating angiogenesis were analyzed by ELISA in MPs-CCM. *p<0.05 vs. C-MPs; **p<0.01 vs. C-MPs in at least 3 samples. Data is represented as the average of each condition (control vs. diabetic) with a standard error of the mean. **C**) roGFP-expressing HUVECs were used as a tool to analyze the HUVEC susceptibility to oxidative stress. Values expressed as mean fluorescence ± SEM of 3 experiments in quadruplicates. *p<0.05 and **p<0.01 vs. C-MPs-CCM. **D**) HUVECs were treated during network formation with 300μmol/L H_2O_2 to test the effect of oxidative stress. MPs-CCM were also supplemented with 300 μmol/L H_2O_2 and added to HUVECs. Network formation was quantified as number of branches. Values expressed as mean±SEM of 4nexperiments in duplicates. *p<0.05 vs. HUVECs. Scale bar 100 μm.

Figure 5. Analysis of pericyte redox status. A) MPs were stained with 5 μmol/L MitoSox and read on a FACSCantoII flow cytometer in the PE channel. Data quantified in the bar graph. Values expressed as mean±SEM of percentages of positive populations. Sample size n=4, *p<0.05 vs. C-MPs. **B**) Transcriptional analysis of the antioxidant genes SOD-1 and catalase. Values are expressed as mean±SEM of 5 experiments. *p<0.05 vs. C-MPs.

Figure 6. NAC restores D-MPs functions. A) Myotube differentiation was evaluated by assessing MyHC-expressing cells. Quantification was performed counting the number of MyHC-expressing cells on the total cells here expressed as bar graphs. *p<0.05 vs. D-MPs in at least 4 samples. Scale bar 50μm. **B**) Cell proliferation measured by BrdU incorporation

assay following 30 min exposure to NAC (1 mmol/L) prior to BrdU addition. Values expressed as mean±SEM of 4 experiments in triplicate per group. *p<0.05 vs.C-MPs; #p<0.05 vs. D-MPs. **C**) Matrigel-assisted HUVEC network formation was analyzed following D-MPs treatment with NAC (1mmol/L for 30 min). Representative microphotographs and bar graphs show the efficiency of network formation as assessed by counting the number of branches. Values are expressed as mean±SEM of 3 experiments in duplicates. *p<0.05 vs: HUVECs, #p<0.05 vs. HUVECs+C-MPs and §.p<0.05 vs. HUVECs+D-MPs. Scale bar 100μm.

Figure 7. Induced p66^{Shc} expression in D-MPs. A) Transcriptional analysis of the ROS sensor protein p66^{Shc}. Values are expressed as mean±SEM of 3 experiments. *p<0.05 C-MPs. **B**) Western blot: C-MPs (lane 1) and D-MPs (lane 2) were compared for the expression of both total p66^{Shc} and serine phosphorylated (Ser36-p-p66^{Shc}) isoforms using anti-Ser36-p-p66^{Shc}-clone 6E10. **C**) Flow cytometry analysis and quantification of the percentage of Ser36-p-p66^{Shc}positive cells are expressed as mean±SEM of 3 experiments. *p<0.05 vs. C-MPs. **D**) Immunofluorescence microscopy analysis of phospho-p66^{Shc} positive MPs. Scale bar 50 μm.

Figure 8. PKCβII inhibitors LY333531 and CGP53353 restore D-MPs functions counteracting p66^{Shc} activation. A) The selective inhibition of kinase PKCβII responsible for the phosphorylation-mediated activation of p66^{Shc} was achieved treating D-MPs with 200nmol/L LY333531 or 2μmol/L CGP53353. Cell functions like myogenic differentiation (**B**), proliferation (**C**) and network formation on Matrigel (**D**) were all recovered following PKCβII inhibition-dependent p66^{Shc} blockade. Values expressed as mean±SEM. *p<0.05 vs. D-MPs and **p<0.01 vs. D-MPs. For Matrigel assay *p<0.05 vs. HUVECs,#p<0.05 vs.

HUVEC+D-MPs and *p<0.05 vs. HUVECs+D-MPs, and n=3. Scale bar 50μm for myogenic differentiation pictures (20X magnification) and 100μm for Matrigel microphotographs (5X magnification). Sample size n=4.